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C50693.

The Patent Office
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1. Your reference CP12/C7-GB2-TCS

2. Patent application number *(The Patent Office will fill in this part)* 9930499.0 24 DEC 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

PHOGEN LIMITED
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U.K.

Patents ADP number *(if you know it)* 7487366002

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention DELIVERY OF SUBSTANCES TO CELLS

5. Name of your agent *(if you have one)*

MR T.C. STANCLIFFE
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"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Patents ADP number *(if you know it)* 7639123001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
	— NO —	

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
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Continuation sheets of this form	—
Description	18
Claim(s)	—
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Priority documents	—
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	—
Request for preliminary examination and search *(Patents Form 9/77)*	—
Request for substantive examination *(Patents Form 10/77)*	—
Any other documents *(please specify)*	—

11. I/We request the grant of a patent on the basis of this application.

Signature T C Stancliffe Date 24 Dec 1999

12. Name and daytime telephone number of person to contact in the United Kingdom

MR T.C. STANCLIFFE
TEL: 01223 - 436508

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Delivery of Substances to Cells

Field of the Invention

This invention relates to aggregated compositions for delivery of substances such as nucleic acids and proteins into cells. The invention relates to such compositions in themselves, and to methods for their manufacture and use.

Background of the invention and prior art

WO 97/05265 (P O'Hare et al.) relates to transport proteins, in particulr VP22 and homologues thereof, and to methods of delivering these proteins and any associated molecules to a target population of cells. This transport protein has applications in gene therapy and methods of targeting agents to cells where targeting at high efficiency is required.

WO 98/32866 (Marie Curie Cancer Care: P O'Hare et al.) discusses coupled polypeptides and fusion polypeptides for intracellular transport, and their preparation and use, e.g. (i) an aminoacid sequence with the transport fuction of herpesviral VP22 protein (or homologue, e.g. from VZV, BHV or MDV) and (ii) another protein sequence selected from (a) proteins for cell cycle control; (b) suicide proteins; (c) antigenic sequences or antigenic proteins from microbial and viral antigens and tumour antigens; (d) immunomodulating proteins and (e) therapeutic proteins. The coupled proteins can be used for intracellular delivery of protein sequences (ii), to exert the corresponding effector function in the target cell, and the fusion polypeptides can be expressed from corresponding polynucleotides, vectors and host cells.

Elliot and O'Hare (1997) Cell, vol. 88 pp.223-233, relates to intercellular trafficking and protein delivery by a herpesvirus structural protein.

All of these documents are hereby incorporated in their entirety by reference and made an integral part of the present disclosure.

Summary and description of the invention

The present invention provides aggregated compositions comprising VP22 protein, and oligonucleotides or polynucleotides.

According to an aspect of the invention, the mixing of oligonucleotides or polynucleotides with VP22 protein can result in association between the nuceotide and protein to form stable aggregates with particle sizes for example in the range 0.1-5 microns e.g. 1-3 microns.

Ratios of between 2:1 and 1:1 of protein to nucleotide are most preferred for formation of aggregates. Higher ratios of protein can be used, but lower ratios are less preferred.

By aggregates we mean associations of molecules forming particles for example particles of 0.1-5 microns in size e.g. of 1-3 micron in size. 'Aggregate' here is not intended to imply a state of denaturation or inactivity: the aggregates usefully contain active protein and/or functionally active oligo- or polynucleotides.

Oligo- or polynucleotides suitable for forming part of the aggregates of the invention can preferably comprise at least 10 bases(nucleotides) and in length can range widely in size (e.g. in the range 10-50 e.g. 20) e.g. they can be about 4 kilobases in size, and they can comprise plasmids, mini-circles of DNA or double stranded DNA, or other functionally active nucleotide sequences. Optionally, the nucleotide sequences can also be associated with a DNA condenser, e.g. protamine sulphate.

The VP22 protein referred to can be the native VP22 protein of HSV1 or HSV2. Alternatively, compositions according to the invention can comprise a protein with a sub-sequence less than the whole sequence of the wild-type VP22 protein, that retains the transport functionality of wild-type VP22 protein. Such a sub-sequence can be, for example, a protein corresponding in sequence to amino acid residues 159-301 of VP22. Native VP22 is believed to form stable multimers readily, either dimers or tetramers. The sub-sequence based on amino acids 159-301 of VP22 is believed to form dimers readily. The VP22 protein, or protein based on a functional sub-sequence, can further comprise other sequences, e.g. at least one flanking tag fused at the N terminus or at the C terminus of the VP22 or sub-sequence. The tag can be for example, a T7 tag which is an example of an epitope tag enabling antibody detection, e.g. at the N terminus, or it can be for example, a his tag which enables purification of the protein on a nickel containing

column, e.g. at the C terminus.

The oligonucleotides or polynucleotides contained in the aggregated composition can be DNA or RNA. When the nucleotides forming the aggregates are RNA, the ribose sugar can be 2'-O-methylated for increased nucleotide stability. In certain examples, the nucleotides can comprise phosphonate derivatives or morpholino derivatives.

In an embodiment of the invention the aggregates can form part of a Streptavidin-biotin complex in which the oligo- or polynucleotide is labelled with biotin, e.g. at the 5' end, and this can then be mixed with streptavidin, e.g. Streptavidin Alexa 594, which is streptavidin bound to a fluorophore molecule. Preferably, the streptavidin molecule is modified so that it can be coupled to a molecule, e.g. a drug, which it is desired to deliver to cells , e.g. so that it comprises a disulphide bond which can be used to link it to a molecule which it is desired to deliver to cells and thereby promote subsequent release of the molecule within the cell by intracellular cleavage of the disulphide bond.

Stability of the nucleotides can be increased by formation of the aggregates according to the invention. The aggregates so formed can be stable in serum, in spite of the presence of Dnases in serum. They can also be stable in high concentrations of denaturants such as urea, e.g. 7M urea.

Where the oligo- or polynucleotides contain phosphorothioate or other modified nucleotide units as mentioned above, they can be especially stable against degradation by components of serum.

The oligo-or polynucleotides contained in the aggregated compositions can contain ordinary nucleotide phosphodiester linkages. Alternatively, e.g. for achieving longer life and stability against hydrolysis, they can contain phosphorothioate linkages in place of phosphodiester linkages.

It can also be useful to label the the oligo- or polynucleotide, for example to facilitate detection and monitoring of the aggregate. The label can be at either the 5' or at the 3' end of the synthetic nucleotide. For detection or monitoring of the aggregate any label capable of detection can be used, such as radio-label, or a fluorochrome label.

The nucleotide can be a fluorescent-labelled 20 base oligonucleotide (20-mer) containing phosphorothioate linkages. It can be labelled at the 5' end with 5' fluorescein phosphoroamidite (Genosys), or at the 3' end with fluorescein (Genosys), or at the 5' end with a terminal fluoresceinyl-base (Life Technologies). Also usable is a Texas Red labelled 20mer phosphorothioate that is labelled at the 5' end with Texas Red (Genosys).

Aggregates according to the invention can be used to deliver their constituents into target cells.

Cells to which the aggregates can be delivered can be cells of a tissue or an organ in a mammalian subject e.g. a human subject, or they can be explanted cells, or they can be cultured cells e.g.for production of a desired protein. Cultured cells that can be used include but are not limited to CHO, COS, HeLa and Vero cells.

In certain examples, when the composition comprises a protein or peptide fused to VP22, or to a sub-sequence thereof, the protein or peptide can be any which can generate an antibody or CTL immune response. Thus the compositions of the invention can be immunogenic compositions, for example they can be vaccines, e.g. DNA or protein vaccines, or both.

In certain examples, the VP22 protein can usefully be a fusion protein in which the protein fusion partner possesses enzymatic activity. For example, a VP22-TK fusion protein, can be used in the compositions e.g. where the target cells are cancer cells e.g. neuroblastoma cells. The compositions can be delivered to target cells, and this can be followed by treatment of the target cells with ganciclovir or equivalent drugs, whereby the TK activity in the composition transported into the cell activates the ganciclovir for cell killing in per se known manner.

It can also be useful to deliver proteins of the compositions for corrective protein therapy.

It can also be useful where VP22, or a sub-sequence thereof, is fused to a cell targeting peptide, such as a peptide that binds to a cell surface receptor, to facilitate cell specific targeting of the complex, e.g. VP22 can be fused to a tumour targeting molecule, such as transferrin or folate.

The oligonucleotide or polynucleotide contained in the aggregated composition according to the invention can be a substance which it is desired to deliver to a target cell.

For example, the oligonucleotide or polynucleotide can be single stranded DNA or RNA, such as a 20mer, and it can have a base sequence that enables it, or its transcription product, to function as an antisense or ribozyme molecule in per se known manner, in effect to suppress functional expression of a chosen gene. For example the polynucleotide can be the synthetic hammerhead ribozyme, or any functional homologues or modifications thereof, which can recognise and cleave c-myb RNA, and thereby inhibit cell proliferation (Jarvis et al., J. Biol. Chem., 1996, 271, 29107-29112).

Alternatively, the oligo - or polynucleotide can be antisense, e.g. antisense to a protein which inhibits apoptosis, such as the Bcl protein, or the oligo- or polynucleotide can have the function of correcting splicing defects. The oligo- or polynucleotides can also usefully be chimeroplasts which can correct mutations.

In other examples, the oligonucleotide or polynucleotide can be single stranded DNA of appropriate sequence to enable it to bind to a specific sequence of DNA in the target cell, by forming a triple helix in per se known manner, to block transcription of the gene to which the nucleotide has bound.

In further examples, the oligonucleotide or polynucleotide can be double stranded DNA and can be of appropriate sequence to function as a binding site that binds a specific transcription factor in a target cell, thereby sequestering the transcription factor in the cell (in per se known manner) and suppressing expression of genes that depend for expression on the sequestered transcription factor.

Alternatively or additionally, the protein contained in the aggregated composition according to the invention can be a substance which it is desired to deliver to a target cell. For example, it can comprise VP22 or a protein comprising sub-sequence thereof, or a fusion protein comprising VP22, e.g. for use as a vaccine.

The aggregated compositions according to the invention can also comprise

further or other substances for delivery to target cells, such as nucleotides, proteins or peptides fused to VP22.

For example, the aggregated composition can comprise and deliver to a target cell linear DNA of a size sufficient to encode a gene. The delivered DNA can also comprise the necessary gene expression elements needed for its expression in the target cell.

In certain examples, the aggregated composition can comprise and deliver single stranded mRNA molecules, of size sufficient to be translated into a protein or peptide, into the cytoplasm of a target cell where the mRNA can be translated into protein or peptide.

In a further aspect of the invention, the VP22 component of the aggregate contains a VP22 sequence and a further component, which can be either the remaining part of a fusion protein, a protein sequence of a desired functionality which it is desired to deliver within the target cell or a nucleotide sequence which it is desired to deliver within the target cell.

The further component can be linked to the VP22 by a cleavage-susceptible amino acid sequence which is susceptible to cleavage by intracellular protease within the target cell. The proteolytic site can be e.g. a site cleaved by a virus encoded protease, such as for example an HIV-encoded protease so that cleavage only occurs in virus infected cells, or alternatively the cleavage site can be one which is only cleaved by a cell-specific protease, thereby enabling delivery to a specific cell type. In this aspect of the invention, the fusion protein or coupling product can be delivered within the target cell and cleaved there by protease to release the coupling partner of the VP22, that is, the chosen protein or the nucleotide.

It can also be useful in certain examples to include a coupled protein product that is only active after cleavage of the coupled product in the target cell.

Fusogenic peptides can also be present in the aggregates according to the invention, e.g. influenza haemagluttinin for selective cell targeting and intracellular delivery.

It can also be useful to modify the oligo- or polynucleotide so that it can be

coupled to a molecule which it is desired to deliver to a cell, for example through a disulphide bridge which can be reduced within the cell and thereby facilitate release of the molecule for delivery.

The aggregates can be delivered to target cells in vivo. such as cells of a tissue or an organ in a mammalian subject, e.g. a human subject. It can for example, be advantageous to deliver aggregates to cancer cells e.g. to introduce an antisense molecule which is of appropriate (per se known) sequence to target a chimeric oncogene, or to suppress a cancer gene, e.g. ras or p53, or to suppress an anti-apoptotic gene such as a member of the Bcl gene family.

The aggregates can be delivered to target cells in vivo, by for example, direct injection into target cells, such as a tumour cell mass, or they can be delivered systemically.

Alternatively, the aggregates can be formulated using per se known methods for topical delivery, e.g. for use as part of a therapy for psoriasis, eczema or skin cancer. Alternatively, the aggregates can be encapsulated into slow release capsules suitable for oral delivery using standard methods well known in the art.

The aggregates can also be associated with other delivery systems, for example they can be coupled to liposomes, such as cationic liposomes, or they can be associated with condensing agents, such as DNA condensing agents, e.g. hydrophilic polymers, e.g. protamine sulphate. They can then be delivered by e.g. direct injection into the target cells, such as tumour cells, or alternatively they can be delivered systemically, e.g. using a catheter based approach, or they can be formulated for topical delivery, nasal delivery or oral delivery.

The VP22 component of the aggregates can be stored for long periods at -70 deg C, for example in a solution of PBS, or alternatively it can be lyophilised and re-constituted before use. The oligonucleotide component of the aggregates can be stored for long periods at - 20 deg C or at 4 deg C, for example in a solution of Tris buffer (pH 7.0). The VP22 and oligonucleotide components can then be mixed at room temperature for at least 10 mins to enable formation of aggregates according to the invention just prior to delivery of aggregates to cells.

The aggregates can be delivered to target cells which are cells cultured in vitro, for example to CHO, COS, HeLa and Vero cells. The cultured cells containing the aggregates can be used, for example, for target validation in in-vitro testing of gene expression products.

In other embodiments, cells treated with compositions according to the invention can be explanted cells and can then be re-introduced in vivo, e.g. into a mammalian subject.

The aggregates can be substantially resistant to trypsinisation of cultured cells containing them. Therefore cells containing the aggregates in culture can be trypsinised prior to use.

In a further aspect of the invention, exposure to light such as fluorescent light can be used to promote more rapid disaggregation of the aggregates. For example, after internalisation of the aggregates, target cells in vitro can be exposed to fluorescent light, and where those cells are in vivo they can be exposed to a laser e.g. during photosurgery.

The aggregated compositions can also comprise a photosensitising molecule, e.g. fluoroscein, rhodamine, or TRITC, which can be linked to the 5' or 3' end of the synthetic nucleotide. This can facilitate the disaggregation of the aggregates in the presence of irradiation, e.g. during phototherapy, for example, as part of a treatment for skin cancer or psoriasis. Irradiation can be achieved in vivo, for example, by introducing into a patient to be treated an endoscope comprising laser optic lines for emitting radiation. Dissociation of aggregates can also be facilitated in the absence of light by introduction of a cleavage site, such as a protease site, or a fusogenic peptide, e.g. the FLU fusion peptide.

Aggregates according to the invention can be useful as cell delivery systems for substances such as proteins or nucleotides, fused with VP22 protein, or a functional part thereof, and can enable delivery into target cells of large amounts of protein or nucleotides.

Following exposure of a cell population to such aggregates, they can be taken up by the cells and the VP22 fusion protein can cause transport to the cell nucleus.

Once the aggregates are taken up into a cell they have been observed in certain examples to remain within the cell for some days, and can also resist cell trypsinisation.

Also provided by the invention is a method of making such aggregates, comprising (a) mixing a VP22 protein or a suitable sub-sequence thereof as mentioned above, optionally fused or covalently coupled to a protein sequence or a nucelotide for delivery to a target cell, with an oligonucleotide or polynucleotide followed by (b) incubating the mix obtained in step (a).

The invention also provides a method for transporting substances into cells, comprising contacting target cells with an aggregated composition according to the invention.

The invention in a further aspect also provides a method of producing/purifying a preparation of the VP22 protein, or a sub-sequence thereof, e.g. a sub-sequence comprising amino acids 159-301 of VP22, comprising treating the protein by affinity chromatography or ion exchange, e.g. using DEAE Sepharose, and (e.g. in a subsequent stage) by purification on a nickel-NTA column.

An example of the invention is described below without intent to limit its scope.

Example 1:

This example concerns preparation of an aggregate comprising (i) a fragment of VP22, herein designated 159-301 protein, and consisting of amino acids 159-301 of the VP22 sequence of HSV2 VP22 protein along with (in this example) a his6 tag at the C-terminal end, (ii) and an oligonucleotide which is a 20mer phosphorothioate (of base sequence CCC CCA CCA CTT CCC CTC TC; from Genosys) labelled at the 3' end with fluorescein.

The 159-301 protein can be prepared for example as follows:

159-301 protein can be made in an E.coli expression system expressing a plasmid encoding 159-301 protein, which is a PET-based plasmid containing an

IPTG sensitive promoter. The his tag is placed at the C terminus of the protein.

50 ml of bacterial culture expressing the plasmid mentioned above can be grown in nutrient broth suitable for the growth of E. coli, such as L nutrient broth (Oxoid), and also containing kanamycin and chloramphenicol. The recombinant bacteria can be induced by addition of IPTG (0.5mM) to a logarithmic phase culture, and the cells harvested by centrifugation (6000rpm, 4 degC, 20 min). After pelleting the cells can be resuspended in 60 ml of cold lysis buffer containing: 50mM sodium phosphate (pH8.0), 300mM sodium chloride, 5mM imidazole (pH 8.0), 5mM beta-mercaptoethanol, 5 microg/ml Rnase and 5 microg/ml of Dnase-I, 0.5mM PMSF, 1microg/ml of leupeptin, 1microg/ml of pepstatin and 1mg/ml of lysozyme.

The lysis mixture is incubated for 30 min with occasional shaking, and is then sonicated on ice three times for 15 seconds followed by addition of 0.1% NP-40. Dnase and Rnase are then added to 10 microg/ml and incubated on ice for 20 min with occasional shaking. The lysate is then drawn through a narrow gauge syringe three times. This is followed by centrifugation of the lysate at 14000rpm for 15 min at 4 degC. The supernatant containing the protein is retained.

The 159-301 protein can be purified as follows:

The protein can be purified by ion exchange chromatography on DEAE sepharose (Pharmacia). The column is centrifuged (3000rpm, 4degC, 5 min) in the presence of lysis buffer comprising 50mM sodium phosphate (pH8), 300mM sodium chloride, 5mM imidazole (pH8), 5mM beta-mercaptoethanol, 5 microgram/ml Rnase and 5 microgram/ml Dnase, 0.5mM PMSF and 10% glycerol, 0.1% NP-40,40mM imidazole (pH8.0), and 1 microgram/ml leupeptin and 1 microgram/ml pepstatin.

The eluate obtained is then further purified on a nickel-NTA column. Unbound protein is collected, and the column is then washed in wash buffer which has the same composition as lysis buffer except that it contains 10% glycerol, 0.1% NP-40,40mM imidazole (pH8.0), and lacks RNase and DNase. Bound protein is then eluted in eluate buffer which has the same composition as lysis buffer except that it contains 10% glycerol, 0.1% NP-40, 500mM imidazole (pH8.0), and lacks

RNase and Dnase.

The 159-301 protein in solution in eluate buffer is used for the formation of the aggregates. Alternatively, it can be dialysed for 12 hours in PBS before use.

Aggregates can be produced as follows:

25 microlitres of 20mer phosphorothioate-linked oligonucleotide as described above (10micromolar solution in PBS) labelled at the 5' end with fluorescein is added to 25 microlitres of 159-301 protein solution in PBS (20 micromolar solution which contains approximately 150mM sodium chloride and 10mM phosphate at a pH between 7 and 7.2). The final concentration of 159-301 protein in 50 microlitres of PBS is about 10 micromolar and the final concentration of oligonucleotide is about 5 micromolar. The mixture is mixed and left at least 10 min at room temperature. Fifty microlitres of this mixture is then added to 450 microlitres of tissue culture medium (with or without added)serum and can be stored at about 4degC.

The formation of the aggregates of the invention can be monitored by using microscopy e.g. phase contrast or fluorescence microscopy, or by agarose gel electrophoresis of the aggregates.

Aggregates can be delivered to cells as follows:

Aggregates produced by the method previously described can be diluted in pre-warmed tissue culture medium and then added to HeLa cells and incubated for about 12 hours at a temperature of 37degC.

Example 2:

An aggregate can be made by a method similar to that described in Example 1, except that the oligonucleotide used in the preparation is a oligonucleotide which is a 40mer phosphorothioate labelled at the 5' end with Texas red and with a base sequence as follows:

5' TCC TCG CCC TTG CTC ACC ATG GTG GCG ACC GGT GGA TCC C 3'

This sequence is commercially available and is complementary to a segment of GFP mRNA.

Monitoring of the formation of the aggregates and delivery of the aggregates to cells can be carried out as described in example 1.

Example 3:

This example is similar to Example 2, except that the oligonucleotide sequence is as follows:

5' CCC TTG CTC ACC ATG GTG GC 3'.

Example 4:

This example is similar to Example 1, except that the oligonucleotide sequence is as follows:

5' ACC ATG GTG GCG ACC GGT GGA TCC C 3'.

Example 5:

This example is similar to Example 1, except in that a) the oligonucleotide sequence is as follows:

5' CCC TTG CTC ACC ATG GTG GC 3',

and b) that the aggregates are added to the cells without dilution in PBS and are incubated with the cells for about 2 hours at a temperature of 37degC.

Example 6:

An aggregate can be made by a method analogous to that described in Example 1, except that (i) the fragment of VP22 consists of amino acids 159-257 of the VP22 sequence of HSV2 VP22 protein, and (ii) the oligonucleotide is a 20mer phosphorothioate labelled at the 5' end with fluorescein and with a base sequence as follows:

5' CCC CCA CCA CTT CCC CTC TC 3'.

This sequence is commercially available and is complementary to a segment of mRNA encoding an intracellular- adhesion molecule, or ICAM.

The 159-257 protein can be prepared and purified as described in Example 1 for preparation and purification of the 159-301 protein, except for the use of an

otherwise corresponding plasmid encoding 159-257 protein.

In the aggregates produced, final concentrations of protein and oligonucleotide in 50 microlitres of solution can be about 13.5 micromolar protein and 5 micromolar oligonucleotide.

Example 7:

An aggregate can be made by a method analogous to that described in Example 1, except that (i) The VP22 '159-301' protein is present as a fusion with the BH3 domain of the bak protein, and (ii) the oligonucleotide is labelled at the 5' end with FITC. A BH3-VP22 '159-301' protein fusion protein can be made as follows:

A double stranded oligonucleotide with the following sequence corresponding to BH3 can be made and cloned into the Bam H1 site of the VP22 '159-301' expression plasmid used to encode the VP22 '159-301' protein, as mentioned above in Example 1:

5'GATCCTATGGGGCAGGTGGGACGGCAGCTCGCCATCATCGGGGACGA
CATCAACCGACGCTATCGG
5'GATCCCGATAGCGTCGGTTGATGTCGTCCCCGATGATGGCGAGCTGCC
GTCCCACCTGCCCCATG

The above strands are complementary such that the sequence of the first strand from the seventh residue (adenine) in the 5' to 3' direction is complementary with the sequence of the second strand from the second residue from the end (thymine) in the 3' to 5' direction.

BL21 E. coli cells can be transformed with this BH3-VP22 '159-301' expression plasmid, and are grown, induced and the cells harvested as described in Example 1.

After harvesting the cells can be resuspended in 40ml of cold lysis buffer containing: 50mM sodium phosphate (pH 8.0), 300mM sodium chloride, 5mM

imidazole (pH 8.0), 5mM beta-mercaptoethanol, 1 microg/ml of leupeptin, 1microg/ml pepstatin and 1 mg/ml lysozyme.

The lysis mixture can be incubated for 30 min with occasional shaking, and is then sonicated on ice three times for 15 seconds followed by addition of 0.1% NP-40. Dnase and Rnase can then be added to 10 microg/ml and incubated on ice for 20 min with occassional shaking. The lysate can then be drawn through a narrow gauge syringe three times. This can be followed by centrifugation of the lysate at 20,000rpm for 15 min at 4degC. The supernatant containing the VP22-BH3 fusion protein can be retained. The BH3-VP22 '159-301' fusion protein can be purified as follows:

The protein can be enriched by ion exchange chromatography on DEAE sepharose (Pharmacia) by using a batch method, in the presence of lysis buffer comprising 50mM sodium phosphate (pH 8.0), 300mM sodium chloride, 5mM imidazole (pH 8.0), 5mM beta-mercaptoethanol, 0.1% NP-40, and 1 microgram/ml leupeptin and 1 microgram/ml pepstatin.

The eluate can then be further purified on nickel-NTA beads in a batch method. Protein can be bound to the beads at 4degC for 1h. The beads can then be washed three times for 30 mins in wash buffer of the same composition as lysis buffer except that it contains 10% glycerol, 0.1% NP-40, 40mM imidazole (pH 8.0). Bound protein can then be eluted three times in 1ml of eluate buffer each time. The eluate buffer can have the same composition as lysis buffer except that it contains 10% glycerol, 0.1% NP-40, 500mM imidazole (pH 8.0). The eluate buffer can then be exchanged by PD-10 sephadex column chromatography into PBS, 10% glycerol, 5mM B-mercaptoethanol.

The BH3-VP22'159-301' fusion protein obtained by the method described above can be used in the formation of aggregated compositions using a method analogous to that described in example 1:

22.5 microlitres of BH3-VP22 '159-301' protein in PBS can be added to 2.5 microlitres of PBS and 0.5 microlitres of the oligonucleotide
The final concentration of BH3-VP22 '159-301' fusion protein can be about 18 micrograms per ml and the final concentration of oligonucleotide is about 500nM.
Monitoring of the formation of the aggregates and delivery of the aggregates to cells can be carried out as described in Example 1.

Example 9:

A p27-VP22 '159-301' fusion protein can be made by a method analogous to that described in Example 8 for making a BH3-VP22 '159-301' fusion protein, except for the use of an oligonucleotide with a sequence corresponding to the p27 sequence (GenBank Accession Number U10906) which can be made and cloned into the Nde I and Bam H1 sites of the VP22 '159-301' expression plasmid.

The p27-VP22 '159-301' fusion protein obtained by the method described above can be used in the formation of aggregates using a method analogous to that described in Example 1:
37 microlitres of p27-VP22 '159-301' protein in PBS can be added to 463 microlitres of PBS and 5 microlitres of the oligonucleotide:

The final concentration of p27-VP22 '159-301' fusion protein can be about 185 micrograms per millilitre and the final concentration of oligonucleotide about 2.5 micromolar.
Monitoring of the formation of the aggregates and delivery of the aggregates to cells can be carried out as described in Example 1.

Example 10:

An aggregate can be made by a method analogous to that described in example 1, except that the oligonucleotide is a 36mer ribozyme which is a 36mer ribozyme as described by Jarvis et al., J. Biol. Chem. 1996, 271, 29107-29112, which can recognise and cleave c-myb and so inhibit cell proliferation, and which is

fluorescein labelled at the 5' end and has the following sequence and can be obtained from Cruachem, Glasgow, UK:
5' GUUUUCCCUGAU GAGGCCGAAAGGCCGAAAUUCUCC 3'.

In this sequence all nucleotides are 2'-0-methyl nucleotides with the exception of the following: U at position U5 (i.e. the fifth U residue counting from the 5' end of the sequence), G at positions G2, G3 and G9, A at positions A1and A8 are 2' hydroxyl (ribo)nucleotides. The U at position U5 indicates 2'-0-allyl uridine, the ribozyme described by Jarvis et al. had a 2'-C-allyl uridine linkage at this position (this being the only difference between the ribozyme described here and that of Jarvis et al.). 5 phosphorothioate linkages are present at the 5' and 3' ends, other linkages are phosphodiester.
Aggregates can be produced by adding the 36mer oligonucleotide to the VP22 '159-301' protein solution in PBS as previously described in Example 1, so that the final concentrations in 50 microlitres of solution can be about 18 micrograms per ml (or alternatively about 32 micrograms per ml) protein and about 500nM oligonucleotide.
Monitoring of the formation of the aggregates and delivery of the aggregates to cells can be carried out as described in Example 1.

Example 11:

An aggregate can be made as described in example 10, except that the oligonucleotide sequences differs as follows: the second G residue (counting from the 5' end) has been changed to 2'-0-methyl uridine, and the seventh A residue (counting from the 5' end) has been changed to 2'-0-methyl uridine.

Example 12:

An aggregate can be made by a method similar to that described in Example 1, except that the oligonucleotide is labelled with biotin at the 5' end and has the following sequence:

5' CCC CCA CCA CTT CCC CTC TC 3', and can be obtained from Sigma Genosys), and the aggregate further comprises streptavidin-Alexa 594, which is a protein-fluorophore, and can be obtained from Molecular Probes, Netherlands.

The aggregates can be prepared as follows: 12.5 microlitres of the biotin labelled oligonucleotide (20 microM in PBS) can be mixed with 12.5 microlitres of streptavidin-Alexa 594 (400 nanoM in PBS) and the mixture incubated for 2 hours at room temperature with occasional stirring. Twenty five microlitres of VP22 protein (360 micrograms per ml in PBS) can then be added to the mixture and this mixture incubated for 10 mins at room temperature.

Alternatively, the aggregates can be prepared as follows: 12.5 microlitres of the biotin labelled oligonucleotide (20 microM in PBS) can be mixed with 12.5 microlitres of VP22 (720 micrograms per ml in PBS) and the mixture incubated for 10 mins at room temperature. Twenty five microlitres of streptavidin-Alexa 594 (200 nanoM in PBS) can then be added to the mixture and this mixture incubated for 2 hours at room temperature.

Formation of the aggregates can be monitored as described in example 1.

Aggregates can be delivered to COS cells using the following method: aggregates can be diluted 10 times in cell culture medium containing 10% serum at final concentrations of about 500nM biotin labelled oligonucleotide, about 10nM streptavidin-Alexa594 and about 18micrograms per ml VP22. The cells can then be incubated with the complexes overnight.

The present disclosure extends to modifications and variations of the description given herein that will be apparent to the reader skilled in the art. The disclosure hereof, incorporating WO 97/05265, WO 98/32866 and Elliot and O'Hare (1997) which are made an integral part hereof, is intended to extend in particular to classes and subclasses of the products and generally to combinations and subcombinations of the features mentioned, described and referenced in the present disclosure. Documents cited herein are hereby incorporated in their

entirety by reference for all purposes.